

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 372

INSULIN SECRETION

Its Regulation by Monoamines and
Acid Amyloglucosidase

By

INGMAR LUNDQUIST

1972

LUND 1974

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SUPPLEMENTUM 372

From the Department of Pharmacology, University of Lund, Sweden

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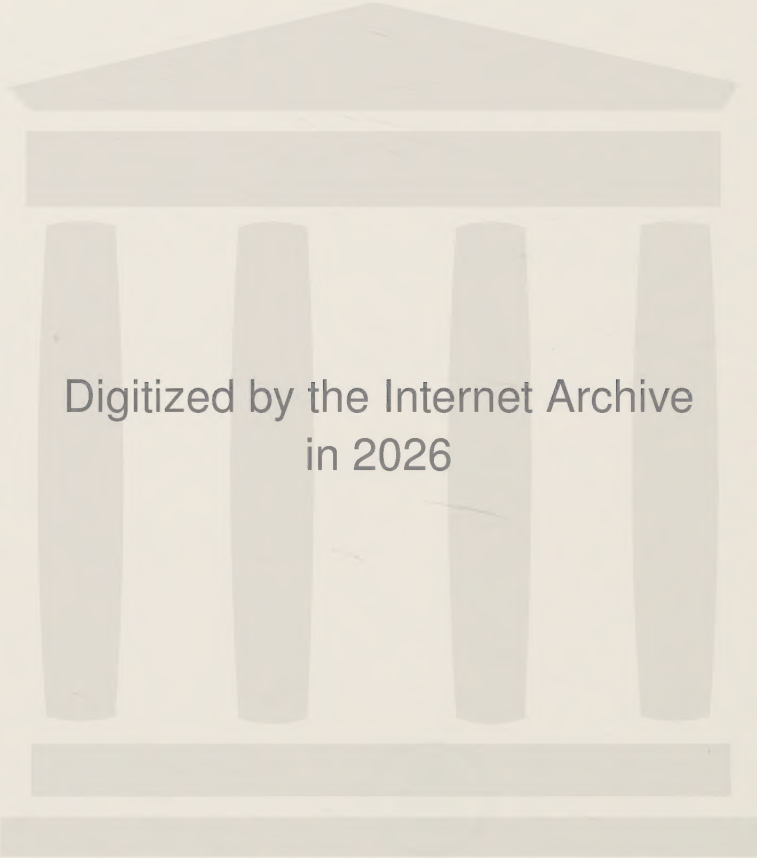
INGMAR LUNDQUIST

Lund 1971



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Studentlitteratur
Lund 1971

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This dissertation summarizes the following papers, which will be referred to by the figures 1-14 given below.

1. C.RERUP and I.LUNDQUIST, Blood glucose level in mice,
1. Evaluation of a new technique of multiple serial sampling,
Acta endocr., 1966, 52, 357-367,
2. C.RERUP and I.LUNDQUIST, Blood glucose level in mice,
2. A quantitative study of alloxan diabetes, Acta endocr., 1967,
54, 514-526,
3. C.RERUP and I.LUNDQUIST, Non-specific reaction of current
glucose oxidase preparations with glycogen and its application
for glycogen determinations in tissue, Acta pharmacol. toxicol.,
1967, 25, 41-53,
4. I.LUNDQUIST, K.LINDSTRAND and C.RERUP, Separation and
characterization of a glycogenolytic enzyme from a glucose oxidase
preparation obtained from Aspergillus niger, Scand. J. clin. Lab.
Invest., 1969, 23, 89-95,
5. I.LUNDQUIST, Method for determination of acid amyloglucosidase
in isolated islets of the pancreas. Submitted for publication in Enzyme.
6. I.LUNDQUIST and C.RERUP, On the development of alloxan diabetes
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7. I.LUNDQUIST, Interaction of amines and aminergic blocking agents
with blood glucose regulation, I. β -Adrenergic blockade, Europ. J.
Pharmacol., (in press).
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with blood glucose regulation, II. α -Adrenergic blockade, Europ. J.
Pharmacol., (in press).
9. R.HÅKANSSON, I.LUNDQUIST and C.RERUP, On the hyperglycaemic
effect of DOPA and dopamine, Europ. J. Pharmacol., 1967, 1, 114-119,
10. R.EKHOLM, L.E.ERICSON and I.LUNDQUIST, Monoamines in the
pancreatic islets of the mouse. Subcellular localization of
5-hydroxytryptamine by electron microscopic autoradiography,
Diabetologia, 1971, 7, 339-348.

11. I. LUNDQUIST, R. EKHOLM and L. E. ERICSON, Monoamines in the pancreatic islets of the mouse. 5-Hydroxytryptamine as an intracellular modifier of insulin secretion, and the hypoglycaemic action of monoamine oxidase inhibitors, Diabetologia, (in press).
12. I. LUNDQUIST, Acid Amyloglucosidase and carbohydrate regulation. I. Effect of exogenous amyloglucosidase on tissue glycogen, blood glucose and plasma insulin, Horm. Metab. Res. (in press).
13. I. LUNDQUIST, Acid amyloglucosidase and carbohydrate regulation. II. Acid amyloglucosidase activity in the endocrine pancreas. Horm. Metab. Res. (in press).
14. I. LUNDQUIST, Acid amyloglucosidase and carbohydrate regulation, III. The induction of sulphonylurea-stimulated insulin release and its dependence on intracellular monoamines, Horm. Metab. Res. (in press).

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I. INTRODUCTION

The regulation of carbohydrate metabolism in mammals is dependent upon the β -cell in the islets of Langerhans because of the specific ability of this cell to synthesize and release insulin. The pancreatic islets are morphologically a discrete group of cells scattered within the exocrine tissue and comprising about 1 % of the total pancreatic mass. In the β -cell, insulin (and/or its precursor) is stored mainly in specific cytoplasmic granules. According to the emiocytosis theory (cf. Lacy 1967, 1970) stimulation of insulin secretion causes migration of the secretory granules towards the inner surface of the cell, where the granules dissolve, after fusion with the cell membrane, and the solubilized hormone enters the vascular compartment. However, other modes of insulin secretion have been suggested, e.g., intracellular liberation of intragranular insulin stores by gradual dissolution of the content of the granule (Orci *et al.* 1969), and also a secretory mechanism which completely by-passes the granules (Like and Miki 1967, Renold 1970).

Although glucose is considered to be the main regulator of insulin secretion, a variety of other substances have been shown to stimulate or to inhibit the release of the hormone. The number of such agents has become considerable during the past few years; for a coverage of these general aspects on insulin secretion, the reader is referred to several recent and excellent reviews (Grotsky and Forsham 1966, Frohman 1969, Mayhew, Wright and Ashmore 1969, Curry 1970, Levine 1970, Renold 1970, Porte and Bagdade 1970, Lacy 1970).

The purpose of this summary is to discuss some new aspects of the insulin secreting processes in mice. Results are presented from studies which show that insulin secretion is partly regulated by the influence of certain arylethylamines and by the action of acid amyloglucosidase. *)

*) Terminology in the field of carbohydrases with exoamylolytic properties is confusing. Exoamylolytic enzymes which attack glucose polymers predominantly linked with α -1,4-bonds, such as oligoglucosides, glycogen and starch, by the stepwise removal of glucose units from the non-reducing ends of the molecule, are generally referred to as glucoamylase (EC 3.2.1.3) or α -glucosidase (EC 3.2.1.20). The term acid amyloglucosidase is preferred by the author to characterize both the activity of the purified fungal enzyme and the activity recorded in isolated pancreatic islets. The term acid amyloglucosidase is not limited to an enzyme capable of splitting only α -1,4-bonds. On the other hand, it does not cover other possible neutral glucoamylases or acid and neutral maltases not operating on glycogen at acid pH.

The work was concerned with the following problems:

The possible influence that extracellular monoamines*) and monoamines in the β -cell may exert on insulin secretion; also the influence of catecholamines on peripheral glucose utilization.

The importance of β -cell glycogenolysis in insulin secreting processes by means of hydrolytic breakdown by acid amyloglucosidase as opposed to the phosphorolytic breakdown by glycogen phosphorylase. The latter reaction has previously been reported to be extremely slow (Matschinsky and Ellerman 1968).

In addition, the insulin secretory capacity and the carbohydrate balance during the development and different stages of alloxan diabetes were investigated to obtain models of different diabetic conditions.

II. MATERIAL

Animals. Female NMRI-mice (Laboratory Animal Breeding, Laven, Denmark and Anticimex, Stockholm, Sweden) or American Obese mice (AO-mice) and their lean litter mates (AN-mice) obtained from Novo Ltd., Copenhagen, Denmark. The animals were kept on a standard pellet diet (Ferrosan AB, Malmö, Sweden, or Astra-Ewos, Södertälje, Sweden) and tap water ad libitum before and throughout all experiments. Adrenalectomy was performed under ether anaesthesia by the lumbar approach. Adrenalectomized animals were kept on the standard pellet diet and given 0.9 % NaCl to drink. Hypophysectomy was performed under ether anaesthesia by the transauricular route (Falconi and Rossi, 1964). Unless otherwise stated, alloxan diabetes was induced in non-fasted animals by the intravenous injection of 0.44 mmoles/kg of alloxan monohydrate.

Chemicals. The sources of the different chemicals:
Glucose oxidase 250 (high purity), "Fermcozyme" CB-B and "Fermcozyme" AM (Hughes & Hughes Ltd., Brentwood, England),

*) Extracellular monoamines, as a term, covers monoamines circulating in the blood as well as noradrenaline released locally from sympathetic nerve endings, as opposed to the intra- β -cell monoamines.

"Agidex" (Glaxo Laboratories Ltd., Greenford, England), Glucose- $U^{14}C$, ^{14}C -5-hydroxytryptamine, ^{14}C -DL-DOPA and 3H -DL-5-HTP (Radiochemical Centre, Amersham, England), Purified shellfish glycogen and EDTA- Na_2 (British Drug Houses Ltd., Poole, England or Merck AG, Darmstadt, Germany), Maltose A.R. (Kebo AB, Stockholm, Sweden or Koch-Light Laboratories, Ltd., Colnbrook, Bucks, England), Cetylpyridinium chloride (Recip AB, Stockholm, Sweden), Protamine hydrochloride (Vitrum AB, Stockholm, Sweden), p-Chloromercuribenzoate (Sigma Chemical Corporation, St. Louis, Missouri, USA), Bio-Gel P 150 (Bio-Rad Laboratories, Richmond, Virginia, USA), Sephadex G 150 and G 25 (Pharmacia AB, Uppsala, Sweden), DEAE-cellulose (Serva, Heidelberg, Germany), Dowex 50 W-X4 (H) 200 - 400 mesh and Polyethylenglycol 20 M (Kebo AB, Stockholm, Sweden), o-Dianisidine base (Fluka AG, Buchs, Switzerland), HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid) and turanose (Nutritional Biochemical Corporation, Cleveland, USA), Bovine albumin (Armour Pharmaceutical Company Ltd., Eastbourne, England), Collagenase (Worthington Biochemical Corporation, Freehold, USA), Insulin immunoassay kits (Novo Ltd., Copenhagen, Denmark). All other chemicals were from British Drug Houses Ltd., Poole, England.

Drugs. The sources of the different drugs: L-adrenaline and L-noradrenaline (Rhône-Poulenc Ltd., Paris, France, or British Drug Houses Ltd., Poole, England). L-3,4-dihydroxyphenylalanine, L-5-hydroxytryptophan, dopamine hydrochloride, 5-hydroxytryptamine creatinine sulphate and 5-hydroxytryptamine hydrogen maleinate (Fluka AG, Buchs, Switzerland), L-isopropylnoradrenaline bitartrate and L- and D-isomers of alprenolol (Hässle AB, Göteborg, Sweden). Ro 4-4602 (N^1 (DL-seryl)- N^2 -(2,3,4-trihydroxybenzyl)hydrazine hydrochloride and alloxan monohydrate (Hoffmann-La Roche Ltd., Basle, Switzerland). Nialamide (Pfizer AB, Stockholm, Sweden). Tolbutamide and glibenclamide (Boehringer Mannheim GmbH, Germany) NSD 1015, m-hydroxybenzylhydrazide (Ferrosan AB, Malmö, Sweden). Tyramine hydrochloride (Kebo AB, Stockholm, Sweden), Reserpine, phentolamine methane-sulphonate and phentolamine hydrochloride (CIBA AB, Stockholm, Sweden). L- and D-isomers of propranolol (ICI Ltd., Macclesfield, England). Glucagon-free pig insulin, 10 times recrystallized, 25 IU/mg (Novo Ltd., Copenhagen, Denmark). Dexamethasone, 9 α -fluoro-16-methylprednisolone, (Fredriksberg Chemical Laboratories Ltd., Copenhagen, Denmark). Pargyline hydrochloride (Abbot Laboratories Ltd., North Chicago, Ill. USA). All other drugs were from British Drug Houses Ltd., Poole, England.

III. METHODS

Applicability of serial blood sampling by orbital puncture for the study of blood glucose fluctuations in mice

At the investigation of factors influencing insulin secretion, it was considered of importance to study the alterations in the blood glucose level. With sensitive methods for blood glucose determination, repeated observations on changes in the blood glucose levels in mice would be possible over a long period. The introduction of the enzymatic technique for blood glucose determinations made it possible to work with blood samples as small as 10-25 μ l (paper 1). Most studies of blood glucose levels in mice had previously been performed by sampling of tail blood (Goetz et al. 1954, Baird and Bornstein 1959, Brodin et al. 1964, amongst others). This sampling procedure, which causes considerable loss of blood, was regarded not only as stressful but also as time consuming. Therefore, for the purpose of multiple serial blood sampling for blood glucose determinations, a technique for puncture of the retrobulbar venous plexus by commercially available constriction pipettes was evaluated (paper 1). The orbital bleeding technique was described already in 1913 by Pettit, and serial blood sampling with this approach was performed by Crofford and Davis (1965). These authors claimed that serial blood sampling with this technique was not accurate because of a "stress" which influenced the blood glucose level. However, it was found (paper 1) that, after practice and strictly controlled animal handling, this technique gave very reproducible results with a precision permitting the detection of blood glucose level changes of about 15 mg/100 ml or more with very high statistical significance in a group of 5 normal mice. The variations observed in the blood glucose levels in adrenal-ectomized and alloxan diabetic animals were, as could be expected, larger than in normal mice (papers 1 and 2). The handling of the animals, the blood sampling, and the injection resulted in changes in blood glucose levels in some of the experiments. These changes, although slight, were sometimes significant owing to the precision of the method. Therefore, in all studies on blood glucose changes, control groups were used and the changes were expressed as the difference between experimental and control values.

Method for tissue glycogen determination

Tissue glycogen changes occur often as a consequence of fluctuations in the plasma insulin level. Of the numerous methods for glycogen

determination reported in the literature, acid hydrolysis of glycogen followed by the determination of glucose (Good, Kramer and Somogyi 1933, Fong, Schaffer and Kirk 1953, amongst others) or the direct reaction of glycogen with the anthrone reagent (Seifter et al. 1950, Carroll, Longeley and Roe 1956, amongst others) have been most frequently used. Acid hydrolysis of glycogen has been combined with different chemical procedures for the determination of the glucose formed. Of the different methods for determining glucose, preference was given to the one using the enzyme glucose oxidase (paper 1) because of its ease of performance, sensitivity, and claimed high specificity (Huggett and Nixon 1957, Middleton and Griffiths 1957, Marks 1959). During the evaluation of a method for the measurement of glycogen by means of acid hydrolysis followed by the glucose oxidase procedure, it was observed that the glucose oxidase reagent reacted quantitatively with glycogen even without hydrolysis. The most likely explanation of this phenomenon was that glycogen was converted directly into glucose by enzyme(s) present in the commercial glucose oxidase reagent. This finding led to the development of a new, rapid, and sensitive method for glycogen determination (paper 3).

In short: The tissue specimen is digested in 30 % KOH in a boiling water bath, the glycogen is fractionated with ethanol (1.5 ml pure ethanol/ml KOH), and the tube is then centrifuged, decanted, and allowed to drain. The precipitated glycogen is dissolved in a suitable volume of water depending on the expected glycogen level, e.g., for liver tissue 1 ml water/10 mg of wet liver, and a small sample, 25 - 100 μ l, is withdrawn and added to 3 ml of glucose oxidase reagent (Marks 1959). The reagent contains crude glucose oxidase, peroxidase, and o-tolidine in an acetate buffer pH 5.0. Purified shellfish glycogen is used as reference standard. Colour development is allowed to proceed for 10 min and the sample is read at 625 nm. Because acid hydrolysis of glycogen did not give total recovery (paper 3) and because the anthrone method gives occasional variations in colour yield of replicate samples (unpublished), the above-summarized method is thought to be more accurate than other available methods. It is worth noting that methods for glycogen measurement based on treatment of a tissue homogenate with commercially available preparations of amylolytic enzymes (cf. Huijing 1970) do not measure glycogen alone, but also oligoglucosides of different chain length.

It was shown later (paper 4) that the crude glucose oxidase preparation ("Fermcozyme" CB-B) contained an amyloglucosidase of high activity. Therefore, this glucose oxidase preparation is recommended for the purpose of glycogen determination (paper 3). Most commercial glucose oxidase preparations currently available are less useful since they are more pure and thus contain very small amounts of amyloglucosidase.

Purification and characterization of fungal acid amyloglucosidase

The presence in crude glucose oxidase preparations of an enzyme (or enzymes) with the ability to split glucose from glycogen is of interest not only for glycogen assays (paper 3) but also because this amylytic type of enzymes present in crude fungal extracts has been shown to penetrate liver cells when given to animals (Cuthbertson, Fleming and Rice 1967) and man (Hug and Schubert 1967). Therefore, it was decided to purify the enzyme(s) from the crude glucose oxidase preparation, "Fermcozyme" CB-B, obtained from Aspergillus niger (paper 4), and later from a crude preparation of amyloglucosidase "Agidex" (paper 12) also obtained from Aspergillus niger. For separating glucose oxidase which has a molecular weight of about 150,000 (Keilin and Hartree 1948) from amyloglucosidase which has a molecular weight of about 100,000 (Pazur and Kleppe 1962), gel filtration on Sephadex G 150 was found to be suitable, especially when the G 150 column was provided with a top layer of G 25 (paper 4). No separation was achieved on Bio-Gel P 150. This discrepancy is interesting as Sephadex, being a glucose polymer, apparently retained the amyloglucosidase through the formation of an enzyme-substrate complex. A similar retention on dextran gels has also been reported for other amylytic enzymes such as pancreatic amylase (Gelotte 1964) and liver α -glucosidase (Auricchio and Sica 1967). The purified amyloglucosidase was found to have an acid pH-optimum (pH 4.6) with both glycogen and maltose as substrate at a concentration of 0.18 % (paper 4). Heat inactivation experiments revealed that the glycogen and maltose hydrolyzing activities decreased in parallel, suggesting the presence of a single enzyme. Furthermore, thin-layer chromatography of glycogen digests showed that glucose was the only low molecular reaction product, which suggested absence of endoamylytic contaminants. Kinetic studies with the purified enzyme using maltose as substrate allowed calculation of a $K_m = 1.22$ mM at pH 4.6. With glycogen, the rate of hydrolysis did not follow ordinary Michaelis-Menten kinetics. The rate of glycogen hydrolysis could be roughly estimated to about 10 times that of maltose hydrolysis. Furthermore, it was found that the enzyme was capable of splitting not only α -1,4-bonds but also α -1,6-bonds, since 95 % of glycogen was degraded upon prolonged incubation. The purified enzyme could be characterized as an acid amyloglucosidase having properties similar to a previously described preparation of amyloglucosidase from Aspergillus niger (Pazur and Ando 1959, 1960). These authors showed that the initial attack of the amyloglucosidase results in the stepwise liberation of glucose units from the nonreducing end of a polyglucoside molecule.

The intention was to use the purified enzyme for pharmacological purposes, i.e. to study its effects after administration to animals (mice). However, the amount of enzyme obtained by purification of glucose oxidase preparations was found to be insufficient for this purpose. Therefore, a purification procedure was devised with "Agidex" as the source of the acid amyloglucosidase (paper 12). The crude enzyme preparation was adsorbed on a DEAE-cellulose column at pH 6.0 and eluted by a discontinuous gradient of increased salt concentration and lowered pH. The constituents of the crude enzyme preparation eluted in the last step (0.05 M citrate-phosphate, pH 2.8, containing 0.5 M KCl) were concentrated and further eluted on a Sephadex G 150 column. In this way a highly purified acid amyloglucosidase was obtained. The enzyme was electrophoretically pure, (polyacrylamide gel, veronal buffer pH 8.6, stained with Coomassie blue) and the only low molecular reaction product found after glycogen digestion was glucose, as judged by thin-layer chromatography on Kieselguhr G plates, butanol/2,6-lutidine/water 6:3:1 v/v/v.

Method for the determination of acid amyloglucosidase activity in isolated pancreatic islets

During the evaluation of the effects exerted by administered acid amyloglucosidase in mice (paper 12), it became of interest to investigate whether there was any endogenous acid amyloglucosidase activity in the pancreatic islets. Therefore, a method was developed for the determination of acid amyloglucosidase activity in isolated mouse islets (paper 5). The procedure used for the preparation of isolated islets was based on that of Moskalewski (1965). Pancreatic slices were incubated with collagenase in a modified Hanks solution, supplied with fructose instead of the recommended glucose. A low concentration of collagenase was used to minimize cell damage. The islets were harvested one by one by means of a cataract knife and a pair of watch-makers forceps, which was used only to "push" the collagenase-liberated islets onto the cataract knife. Small petri dishes on black paper containing Hanks solution at +4° C and a stereo-microscope were used. Before examination, the islets were washed repeatedly in the modified Hanks solution to remove all collagenase. The islets were transferred to another petri dish for a further washing and then homogenized in a Potter-Elvehjem tube containing 300-500 μ l of an acetate-EDTA buffer (1.1 mM EDTA, 5 mM acetate, pH 5.0). Assay of acid amyloglucosidase activity was then performed in small conical test tubes containing 25 μ l homogenate, 25 μ l glycogen-buffer solution, and 5 μ l Triton X-100. The assay conditions were as follows: temperature 37°, glycogen concentration 1 % (w/v), 0.05 M

sodium acetate buffer, 0.5 mM EDTA, 0.5 % Triton X-100, 0.25 M mannitol, pH 5.0.

Glycogen was preferred to maltose as substrate in the assay for several reasons. Glycogen is, by definition, a specific substrate for an amyloglucosidase, though up to now all mammalian amyloglucosidases described have been shown also to split maltose (cf. Smith, Taylor and Whelan 1968). However, it was observed (paper 5) that islet glycogen hydrolyzing activity and islet maltose hydrolyzing activity had different pH-patterns after heat inactivation, which might suggest the presence of two different enzymes preferentially attacking either glycogen or maltose. Thus changes in glycogen hydrolyzing activity do not necessarily involve similar changes in maltose hydrolyzing activity. Another difference between the two substrates was that maltose in contrast to glycogen caused substrate inhibition of enzyme activity at concentrations of 0.5 % and higher (paper 5). Preliminary experiments had revealed endoamylolytic activity in the islets (unpublished); therefore EDTA was included in the assay to prevent the influence of endoamylolytic activity. All endoamylolytic enzymes are believed to be calcium metallo-enzymes (cf. Greenwood and Milne 1968); therefore this precaution was considered appropriate, although no influence of EDTA was normally seen at pH 5.0. A marked inhibitory effect of EDTA on endoamylolytic activity, however, was recorded at pH 5.5 - 6.3 (paper 5). Triton X-100 markedly enhanced the activity of the enzyme, because this detergent makes available the full activity of this particle-bound enzyme (paper 13). Mannitol was included in the assay procedure because it improved the homogenate dilution curve, which for unknown reasons was recti-linear only within a rather narrow range (paper 5). One unit of acid amyloglucosidase activity is defined as the activity that liberates 1 μ mole glucose/min from glycogen under the above-mentioned assay conditions. An activity of about 15 units/g of protein was found in islet tissue of the non-fasted NMRI-mouse. This activity was found to be twice that recorded in liver tissue (paper 13).

Blood glucose determination. Determination of blood glucose levels was routinely performed enzymatically (glucose oxidase) according to Marks (1959) with a minor modification consisting of a reduction in volume of the deproteinizing fluid (NaOH + ZnSO₄ in NaCl) to a total volume of 2 ml.

Assay of immunoreactive insulin. Radioimmunological determination of insulin in plasma or pancreatic extracts was performed according to Heding (1966) using ¹²⁵I-labelled pig insulin and guinea pig anti-pig-insulin.

In vitro glucose utilization by isolated tissues. Liver, muscle, and adipose tissue specimens were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) supplied with 2 mg gelatine, 2 mg glucose, and $0.1 \mu\text{C}$ (liver $0.3 \mu\text{C}$) glucose- $\text{U-}^{14}\text{C}$ per ml of incubation medium. The incubations were performed in a metabolic agitator (100 cycles/min) in an atmosphere of 95 % O_2 and 5 % CO_2 . The amount of labelled CO_2 formed was determined according to Lyngsøe (1961), ^{14}C -lipid was isolated according to Fain, Scow and Chernick (1963), ^{14}C -glycogen in adipose tissue according to Leonards and Landau (1960), and ^{14}C -glycogen in liver and muscle according to the above described procedure (KOH-ethanol fractionation) but the isolated glycogen was precipitated twice.

Determination of monoamine oxidase and DOPA decarboxylase activity. These activities were determined in pancreatic homogenates (0.1 M phosphate buffer, pH 7.0) with the radiometric methods described by Håkanson and Owman (1965) and Håkanson (1966).

Determination of dopamine. Tissue dopamine was measured fluorometrically (Carlsson and Waldeck 1958) after purification by ion exchange chromatography (Bertler, Carlsson and Rosengren 1958).

Determination of protein. Protein was determined according to Lowry, Rosebrough and Farr (1951). Bovine serum albumin was used as standard.

Electron microscopy. In the anaesthetized animal, the pancreas was fixed by perfusion with 50 ml glutaraldehyde via the ascending aorta. The perfusion solution consisted of 3 % glutaraldehyde buffered with 0.075 M sodium cacodylate, pH 7.2. Pancreatic specimens were dissected out from the splenic part of the pancreas, cut into small pieces, and transferred to 1 % osmium tetroxide in blood-isotonic veronal acetate buffer, pH 7.2. Postfixation lasted for 2 h. After dehydration in ethanol, the tissue was embedded in Epon and islet tissue was identified by light microscopy in 1μ sections. For autoradiography, pale golden sections (about $1200 \text{ \AA}$) were cut on an LKB Ultratome, picked up on Formvar-coated copper grids, stained with uranyl acetate and lead citrate, and then covered with a layer of carbon by vacuum evaporation. The emulsion, Ilford L4, was applied to the sections by means of a wire loop (Caro and van Tubergen 1962; Maunsbach 1966). Most grids, and all grids used for quantitative analysis of silver grain distribution, were developed after 6 weeks in Kodak D 19 B (2 min) and fixed in Kodak F-24 fixer (2 min).

IV. EXPERIMENTAL DIABETES INDUCED BY ALLOXAN

In the search for models of different diabetic conditions in mice, the carbohydrate balance of animals during acute diabetogenesis and during manifest diabetes due to alloxan was studied by determination of blood glucose, plasma insulin, and tissue glycogen (papers 1, 2, 6, and 12).

A. The carbohydrate balance during the acute diabetogenic phase

The diabetogenic effect of alloxan has been the subject of numerous investigations ever since the discovery that it can produce a selective necrosis of the islet β -cells (Dunn, Sheehan and McLetchie 1943). (For a recent review, see Rerup 1970.) In normal animals a diabetogenic dose of alloxan is known to elicit an acute triphasic alteration of the blood glucose level consisting of a) an initial hyperglycaemia, b) a transitory hypoglycaemia, and c) a permanent hyperglycaemia. This was found to apply also in mice (papers 2 and 6). The mechanism responsible for the two first phases was unknown for a long time after its discovery. The initial hyperglycaemic phase was interpreted by Dunn et al. (1943) as partly a consequence of insulin deficiency, but Houssay, Orías and Sara (1945) explained it as a direct glycogenolytic effect on hepatic glycogen stores. The present data (papers 2 and 6) show that it is due both to liver glycogenolysis and to an immediate cessation of insulin secretion. The ensuing hypoglycaemic phase was interpreted by Houssay, Orías and Sara (1945) to be due to low glucose production by the liver. Hughes, Ware and Young (1944), on the other hand, suggested that this phase was due to insulin leakage from the damaged β -cells. It was shown (Howell and Taylor 1967, paper 6) that this phase was the result of increased plasma insulin levels. This could also be demonstrated indirectly through the injection of anti-insulin serum which abolished the hypoglycaemic phase (paper 6). The marked increase in liver glycogen levels during this phase also points to an effect of insulin (paper 6). The early permanent hyperglycaemia following the hypoglycaemic phase was characterized by the absence of, or by very low levels of, plasma insulin (paper 6) due to the selective destruction of the β -cells. Despite a highly diabetic condition, as judged from the blood glucose levels, certain animals still had detectable plasma insulin levels showing that not all β -cells were destroyed. The results obtained (papers 2 and 6) suggested the following models of experimental diabetes with regard to the carbohydrate balance:

- 1) The early part of the acute hyperglycaemic phase, i. e. 5 to 30 min

after the intravenous injection of alloxan, is a model for a β -cell, which is totally unresponsive to insulin releasing agents such as tolbutamide (paper 6) and corticotrophin (Lundquist and Rerup 1967), and which has a severely impaired basal insulin secretion (paper 6). It is a transitory diabetic stage with hyperglycaemia and low glycogen levels (papers 2 and 6).

2) The hypoglycaemic phase and the early phase of permanent diabetes represent a condition of profound shifts in the carbohydrate balance chiefly as a consequence of uncontrolled leakage from the damaged β -cells resulting in fluctuations in plasma insulin, blood glucose, and liver glycogen levels (papers 2 and 6).

B. The carbohydrate balance during the chronic diabetic phase

Alloxan diabetes in mice was regarded as manifest if blood glucose levels were permanently elevated above 200 mg %. In addition to the high blood glucose levels and usually non-measurable plasma insulin levels (papers 2 and 6) liver glycogen was decreased as was muscle glycogen (papers 6 and 12). Other signs of diabetes, such as glycosuria, polydipsia, and polyuria, could be registered. The severity and duration of the permanent diabetic phase in mice was found, as could be expected, to depend on the dose of alloxan and on the individual susceptibility to the drug (paper 2). The blood glucose level was relatively constant in individual mice during several weeks of the permanent phase but highly unstable with regard to external influences (paper 2 and unpublished).

Hypophysectomy or adrenalectomy greatly reduced the survival time of diabetic animals (paper 2). The dose of alloxan generally used (0.44 mmoles/kg intravenously) was effective in female non-fasted NMRI-mice and caused permanent diabetes in practically all these animals without any serious side-effects. It is worthy of note that male animals were less susceptible to alloxan and required a larger dose (unpublished). This sex difference in alloxan sensitivity is also known from other species (Beach, Bradshaw and Blatherwick 1951). The permanent diabetic phase was stable in most mice for several weeks (paper 2), however, after about 2 months, a spontaneous remission of the diabetic condition could occur (Rerup 1968). The improvement of the diabetic state coincided with a restoration of the islet β -cell population (Bunnag, Warner and Bunnag 1967). This was taken advantage of to obtain a colony of diabetic and subdiabetic animals with a significant number of functioning β -cells (paper 12).

The results obtained (papers 2, 6, and 12) suggested the following models

of experimental diabetes with regard to the carbohydrate balance:

1) The first 4 - 5 weeks following establishment of the permanent diabetic phase are considered as a useful model of a diabetic condition characterized by high blood glucose levels and severely impaired insulin secretion (papers 2 and 6), unresponsiveness to the hypoglycaemic action of corticotrophin (Lundquist and Rerup 1967), and low tissue glycogen levels (papers 6 and 12), although susceptible to the extrapancreatic effect of a large dose of tolbutamide (paper 6). This model may to some extent resemble juvenile human diabetes.

2) The phase of spontaneous remission of the diabetic state (from about 8 weeks after alloxan injection) is characterized by a significant number of functioning β -cells (Bunnag, Warner and Bunnag 1967, Rerup 1968, paper 12) and a diabetic blood glucose level (Rerup, unpublished, paper 12). With regard to the carbohydrate balance, this model may, to some extent, resemble maturity-onset human diabetes.

V. MONOAMINES AND INSULIN SECRETION

A. Extracellular monoamines, adrenergic receptors, and carbohydrate regulation

As long ago as 1930, Colwell and Bright suggested that infusion of adrenaline will inhibit insulin release in man. The inhibitory effect of adrenaline on insulin secretion, however, was not convincingly demonstrated until a few years after the introduction of the first radioimmunoassay of plasma insulin (Yalow and Berson 1960). Coore and Randle (1964) showed an inhibitory effect of adrenaline on insulin secretion by pancreatic slices in vitro, and Porte et al. (1966) and Kris et al. (1966) reported that adrenaline infused in vivo could similarly inhibit glucose-induced insulin secretion. Moreover, noradrenaline infused in vivo (Porte and Williams 1966) and dopamine added in vitro (Wong et al. 1967) could be shown to inhibit glucose-induced insulin release. The hyperglycaemic effect of dopamine in vivo was also, on indirect evidence, interpreted as partly due to inhibition of insulin secretion (paper 9). Numerous studies have since convincingly demonstrated that the catecholamines are able to inhibit insulin secretion induced by a variety of stimuli (cf. Porte 1969).

According to present views, the basal insulin secretion is regulated

mainly by the blood glucose acting on the β -cell. However, other mechanisms cannot be ruled out, as Porte and Bagdade 1970, and Freinkel, Mager and Vinnick 1968 did not find any relation between the basal plasma insulin levels and the basal blood glucose levels. Similar results were recently found in mice (unpublished) and had previously been noted with regard to serum insulin-like activity (fat pad method) and blood glucose level in the rat (Lundquist 1968). In adrenalectomized rats, however, there was a significant correlation (Lundquist 1968). The possible implication of the adrenals in the basal regulation of insulin secretion was thus considered, and as the pancreatic β -cell has been shown to have both α - and β -adrenergic receptors (cf. Porte 1969) it was adopted as a working hypothesis that adrenaline has a dual action on insulin secreting mechanisms analogous to its effect on the vascular system; i. e. not only an inhibitory action (α -receptor stimulation) on insulin secretion, as has been shown by several workers (cf. Porte 1969, Mayhew, Wright and Ashmore 1969), but also a stimulating one (β -receptor stimulation) (papers 7 and 8).

L-propranolol, a specific β -blocking agent decreased the basal plasma insulin levels in normal mice to about 50 % (paper 7). No effect was recorded with its structural and optical isomer D-propranolol, which is practically devoid of β -blocking properties (Howe and Shanks 1966). Reduction of basal insulin secretion after treatment with racemic propranolol was observed by Gagliardino et al. 1970 a and Werrbach et al. 1970, whereas no change was found by Cerasi, Effendic and Luft (1969), Allison et al. (1969), and Åkerblom, Martin and Cingolani (1969). This discrepancy might partly be explained by the use of racemic propranolol in these studies. The nutritional status of the studied individuals might also be of great significance, because fasting, which was used in some of the above-cited studies, increases the sympatho-adrenal tone, and thus, in the author's opinion, the threshold for α -adrenergic stimulation could already have been reached before the initiation of the experiment, and the promotion of insulin secretion through β -adrenergic stimulation would be masked. This is analogous to the well-known adrenergic effects on the vascular system, where β -adrenergic vasodilation elicited by small concentrations of adrenaline, "adrenaline reversal", changes into a vasoconstriction once the threshold for stimulation of the less sensitive but predominant α -adrenergic receptors has been reached. As a consequence, the β -adrenergic effect will be completely masked. However, a dual adrenergic effect on insulin secretion does not exclude the possibility that other factors, for example, the basal blood glucose levels, might exert a regulatory influence on basal insulin secretion. Moreover, β -adrenergic blockade never abolished basal insulin secretion and systemic infusion or injection of adrenaline usually revealed no

decrease in basal plasma insulin levels (papers 7 and 8, cf. Porte 1969). Kris et al. (1966) and Miller and Soeldner (1969) reported on a decrease of basal plasma insulin levels during adrenaline infusion. However, a noxious effect of high levels of adrenaline on the β -cells cannot be excluded (Loubatières et al. 1965) especially as there is reason to believe that the immediate cessation of insulin secretion after alloxan administration (paper 6) has mechanisms in common with certain effects of the catecholamines (Cohen and Bitensky 1969).

The concept of a dual action of adrenaline on insulin secreting mechanisms was further investigated (paper 8) by means of the α -adrenergic blocking drug phentolamine. It was found that the administration of phentolamine to normal mice induced a marked and long-lasting hypoglycaemia and a significant increase in the plasma insulin levels (paper 8). This increase in the plasma insulin levels after phentolamine administration has previously been observed in various mammalian species (Frohman, Ezdinli and Javid 1967, Buckler et al. 1969, Werrbach et al. 1970, Hertelendy et al. 1970, Gagliardino et al. 1970 a). On the other hand, Porte (1967), Cerasi, Effendic and Luft (1969) and Buse et al. (1970) reported unchanged plasma insulin levels. This discrepancy is probably due to the fact that quite large doses of phentolamine are needed for a complete α -blockade (Nickerson 1965). The increase in basal plasma insulin levels following α -adrenergic blockade is generally interpreted as a blockade of the inhibitory action of the catecholamines on the β -cell. However, even in the presence of phentolamine, L-propranolol decreased the basal plasma insulin levels (paper 8). This finding favoured the hypothesis that adrenaline may have a stimulating influence (through β -adrenergic activation) on basal insulin secreting mechanisms. Because noradrenaline was found to have a stronger insulin releasing effect than adrenaline under α -adrenergic blockade (paper 8), the question arose whether noradrenaline stimulated both α - and β -adrenergic receptors and thus could have the same inherent ability as adrenaline to stimulate insulin secretion under physiological conditions. However, after removal of the adrenal glands, there was no increase in the plasma insulin level after α -adrenergic blockade either in saline pretreated animals or in glucocorticosteroid substituted animals (paper 8). Moreover, the basal immunoreactive insulin levels were reduced after adrenalectomy (paper 8). A similar decrease in serum insulin-like activity (fat pad method) has previously been observed in the adrenalectomized rat (Lundquist 1968). Therefore, from the above data, it seems reasonable to suppose that adrenaline in fact has a dual action on insulin secreting mechanisms. This does not exclude the possibility that local sympathetic activity in the pancreas exerts an inhibitory effect on insulin secretion.

During the studies of adrenergic blockade in mice, it became evident that blockade by L-propranolol and phentolamine, respectively, induced changes in the carbohydrate balance of the animals which could not be explained solely through effects on insulin secretion or through other known effects of adrenergic blockade (cf. Himms-Hagen 1967, papers 7 and 8). These findings will be briefly discussed.

Adrenergic blockade in liver tissue. Studies on adrenergic receptors influencing glucose utilization in liver tissue have been the object of much controversy (cf. Himms-Hagen 1967). This is probably due to species differences. Experiments in mice (papers 7 and 8), although in no way conclusive, suggest that adrenergic regulation of hepatic glycogen metabolism in this species is mediated by both α - and β -adrenergic receptors, although α -adrenergic effects seem to predominate. In vitro incubation of liver slices in the absence or presence of various concentrations of L-adrenaline or L-adrenaline + insulin with either L-propranolol, β -blockade, or phentolamine, α -blockade, showed a glycogen preserving effect of phentolamine as judged from total glycogen content and ^{14}C -incorporation into glycogen from glucose- $\text{U-}^{14}\text{C}$, whereas L-propranolol was almost without effect, although there was a tendency for a slight inhibition of adrenaline-induced glycogenolysis (papers 7 and 8). However, later experiments in vivo (Lundquist 1971) revealed that treatment of mice with doses of insulin that depleted glycogen was counteracted by pretreatment with phentolamine + L-propranolol, whereas injection of either compound alone produced only partial antagonism. In vivo experiments with L-propranolol showed that this drug slightly diminished the hepatic glycogen concentration and rendered the liver cell more susceptible to further glycogen depletion by exogenous catecholamines (paper 7). This was interpreted in the light of the hypothesis proposed by Hetenyi (cited from Pinter et al. 1967): that the β -adrenergic blockade may cause a relative preponderance of α -receptor mediated glycogenolysis. However, the decreased secretion of insulin induced by L-propranolol (paper 7) is perhaps of greater significance in this context because variations in the amount of insulin reaching the liver are liable to influence the hepatic glycogen content (cf., e.g., paper 6).

Adrenergic blockade in muscle tissue. As reported previously in many species (cf. Himms-Hagen 1967), adrenergic regulation of glycogen metabolism in mouse muscle was found to be entirely β -receptor mediated (paper 7). However, despite decreased plasma insulin levels after L-propranolol treatment, there was an increase and not only a preservation of muscle glycogen content in the presence of exogenous catecholamines. This was first interpreted solely as a consequence of a decreased

intracellular pool of glucose-6-phosphate after the β -blockade of muscle glycogenolysis that permitted an augmented glucose utilization. However, further experiments (paper 7) showed that incubation of muscle tissue, either after L-propranolol treatment *in vivo* or after addition *in vitro*, increased $^{14}\text{CO}_2$ production from glucose- $\text{U-}^{14}\text{C}$ compared with control tissue. This finding favoured the existence of a β -receptor in the muscle with the ability to facilitate glucose transport independent of the β -effect exerted through the glycogen phosphorylase-synthetase system (cf. Sutherland and Robison 1969). Thus the hypoglycaemic effect of β -adrenergic blocking drugs, such as propranolol, which is seen in fasting or diabetic individuals (for references see paper 7), can be explained not only through a decreased gluconeogenesis in the liver and decreased availability of free fatty acids (Abramson and Arky 1968) but also through a marked increase of glucose utilization in muscle tissue by the combined effect of a decreased glycogenolysis and a facilitated glucose uptake.

Adrenergic blockade in adipose tissue. The effects of adrenergic blockade on carbohydrate regulation in adipose tissue of mice can be summarized as follows (papers 7 and 8): Adrenergic regulation of adipose tissue glycogen was found to be β -receptor mediated. *In vitro* experiments on the adrenergic regulation of glucose utilization by adipose tissue provided data, which to some extent were similar to those of adrenergic regulation of insulin secretion, i.e. α -adrenergic stimulation inhibited and β -adrenergic stimulation promoted glucose utilization. The recent suggestion by Himms-Hagen (1970) that the lipolytic response of adipose tissue to adrenergic stimulation is regulated by both α -adrenergic receptors which inhibit and β -adrenergic receptors which promote lipolysis agrees with this concept. However, with regard to the interaction of the mechanisms regulating glucose utilization and lipolysis in adipose tissue, it is difficult to draw any definite conclusions from the present experiments.

B. Intracellular monoamines in the pancreatic β -cell

In 1963, Falck and Hellman showed that the pancreatic islets of the duck, guinea pig, cat, dog, and horse contained monoamines demonstrable by their formaldehyde-induced fluorescence (Falck *et al.* 1962). These amines, later identified as 5-hydroxytryptamine (5-HT) and dopamine (Cegrell, Falck and Hellman 1964, Cegrell 1967), were shown to be present in the pancreatic islets of many other species, including man (cf. Cegrell 1968). But the islet cells of the adult mouse were devoid of formaldehyde-induced fluorescence (cf. Cegrell 1968). This, however, does not exclude the possibility that other amines not detectable with available histochemical methods, or low levels of 5-HT and dopamine

normally exist in mouse pancreatic islets, because the mouse islet cells were found to be equipped with the required mechanisms for uptake and decarboxylation of amine precursors and for amine storage and oxidation (cf. Cegrell 1968).

A preliminary attempt was made to study the possible functional implications of these findings by investigating some effects of L-DOPA (L-3, 4-dihydroxyphenylalanine) and dopamine on the blood glucose regulation in normal and alloxan diabetic mice (paper 9). It was observed that both compounds elicited a rapid rise in the blood glucose level of normal mice with a peak after 15 min. The effect of dopamine vanished completely after 30 min, whereas the effect of L-DOPA persisted. The hyperglycaemic effect of DOPA was described already in 1927 (Hirai and Gondo 1927). With the aid of a decarboxylase inhibitor (NSD 1015) and a monoamine oxidase inhibitor (nialamide) it could be demonstrated that the hyperglycaemic effect of DOPA could be ascribed to dopamine or to a dopamine derivative, because the L-DOPA-induced hyperglycaemia was greatly reduced after pretreatment with NSD 1015, whereas pretreatment with nialamide increased the hyperglycaemic response (paper 9). It was also found that the dopamine-induced hyperglycaemia was, at least partly, pancreatic in origin because dopamine, in contrast to adrenaline (paper 7), had no effect on liver glycogen content and also failed to raise the blood glucose level in alloxan diabetic animals. Moreover, tolbutamide antagonized the hyperglycaemic effect of dopamine pointing to a possible interference with insulin release. However, with regard to the rapidity of the hyperglycaemic response elicited by dopamine, there is reason to believe that this response can be partly attributed to an immediate inhibition of peripheral glucose utilization similar to that exerted by adrenaline (paper 7). This presumption is based on the observation (paper 6) that anti-insulin-induced hyperglycaemia was relatively slow in onset.

The uptake of dopamine by the pancreatic islets is poor compared to that of its precursor L-DOPA (cf. Cegrell 1968). Thus, although the hyperglycaemic effect of dopamine was considered to be partly extra-pancreatic, it was still possible that the prolonged hyperglycaemia elicited by L-DOPA and the impaired dopamine-storing capacity in the pancreas of alloxan diabetic mice (paper 9) reflected a role for β -cell monoamines in the mechanism of insulin release. Autoradiographic investigations with the light microscope have revealed a specific uptake of the amine precursors 5-hydroxytryptophan (5-HTP) and DOPA in the pancreatic islets of the mouse (Gershon and Ross 1966, Ritzén, Hammarström and Ullberg 1965, Tjälve 1971). However, studies by these authors at the light-microscope level furnished limited information about the

precise cellular and subcellular localization of the amines, which is considered of importance for the understanding of the possible physiological significance of monoaminergic mechanisms operating in the islet cells. A previous electron-microscope; histochemical study suggested that endogenous 5-HT in the guinea pig β -cells was associated with the secretory granules (Jaim-Etcheverry and Zieher 1968). Therefore a study was undertaken to investigate the subcellular localization of the labelled monoamine ^3H -5-HT, formed from the administered precursor ^3H -5-HTP, in mouse islet cells by means of electron-microscope autoradiography (paper 10). It was found that the autoradiographic silver grains, which represent the labelled compound, appeared over β -cells and α_2 -cells (glucagon producing cells) whereas other islet cell structures were almost devoid of label. Pretreatment with a monoamine oxidase inhibitor, pargyline, increased the amount of silver grains, whereas pretreatment with reserpine or an inhibitor of aromatic amino acid decarboxylase (unpublished) abolished the appearance of silver grains. From this finding, it was presumed that most of the silver grains represented the amine, 5-HT, and not the amine precursor or any metabolite of the amine. Such a presumption is further corroborated by the finding (cf. paper 10) that the technical procedure used for tissue fixation washed out the major part of 5-HTP and amine metabolites, such as 5-HT-o-glucuronide. Furthermore, quantitative analysis of autoradiographic sections revealed that the concentration of silver grains over the specific secretory granules of β -cells and α_2 -cells was 5-10 times higher than over the remaining parts of these cells (paper 10). In the β -cells, the highest relative grain count was recorded 1 h after label injection. The time pattern in α_2 -cells was different and showed the highest relative grain count when first studied (20 min). This time pattern, together with the finding that monoamine oxidase inhibition was considerably more efficient in the α_2 -cells (paper 10), suggests the presence of a higher monoamine oxidase activity in the α_2 -cells.

The morphological finding of a close association between 5-HT and the secretory granules of the β -cell suggested a possible physiological role for the amine in insulin storing or insulin secreting processes (paper 10). Previous investigations on the possible functional role of 5-HT for insulin-secreting mechanisms had been mainly concerned with the effect of added, extracellular 5-HT on pancreatic slices. Thus, Feldman and Lebowitz (1970 a, 1970 b, 1970 c) found that 5-HT inhibited both basal and glucose-mediated insulin release in golden hamster pancreatic slices in vitro, whereas no effect was observed in mouse pancreatic slices. Telib et al. (1968) reported that 5-HT could stimulate insulin release in rabbit pancreatic slices, and Gagliardino et al. (1971) reported similar results with rat pancreatic slices. The results obtained in the present

study (paper 11) were concerned with intracellular effects of 5-HT in the β -cell under in vivo conditions. The amine precursor L-5-HTP was administered 1 h before the experiments, because the 5-HT stores associated with insulin secretory granules were assumed to be maximally loaded at this time (paper 10).

Pretreatment of mice with L-5-HTP markedly reduced insulin release after stimulation with the sulphonylurea compound glibenclamide or the β -adrenergic agonist L-isopropylnoradrenaline (L-IPNA) (paper 11). On the other hand glucose-stimulated insulin release was unaffected even after combined pretreatment with L-5-HTP + the monoamine oxidase inhibitor nialamide. The failure of L-5-HTP to inhibit glucose-induced insulin release in vivo (paper 11) was contrary to very recent in vitro results: Lernmark (1971) found that glucose-stimulated insulin release from microdissected islets of obese hyperglycaemic mice pretreated with nialamide + 5-HTP was completely abolished. Similar results were obtained by Tjälve (1971) after the addition of 5-HTP to rabbit pancreatic slices. Such a discrepancy might be due either to differences in the concentration of the inhibitor or to factors influencing the insulin response in vivo, or also possibly to species differences. However, it has recently been shown (Lundquist, Ekholm and Ericson 1971) that glucose-induced insulin release in vivo was significantly decreased after pretreatment with L-DOPA in a dose equimolar to the dose of L-5-HTP used in the present study (paper 11). This could suggest that dopamine is a more potent inhibitor of glucose-induced insulin release than 5-HT is. The observation that treatment of mice with a monoamine oxidase inhibitor decreased glibenclamide-induced and L-IPNA-induced insulin release favours the supposition that an endogenous amine might actually reside in the mouse β -cells, although not detectable by conventional fluorescence microscope techniques (paper 11).

The combined treatment with a monoamine oxidase inhibitor and L-5-HTP abolished L-IPNA-stimulated insulin release, whereas glibenclamide-induced insulin release could never be totally suppressed (paper 11). This observation, together with the discrepancy in the time of onset of insulin release between these two agents, suggests a different mode of action on the β -cell. Recent experiments (Lundquist, to be published) showed that L-IPNA-induced insulin release is not mediated by lysosomal activation as is the glibenclamide-induced insulin release (paper 14), which further strengthens the above-mentioned suggestion.

The question of whether basal insulin secretion is influenced by intracellular amine levels was not unequivocally answered (paper 11). Treatment with a monoamine oxidase inhibitor tended to decrease basal

plasma insulin levels, and a combination of L-5-HTP and a monoamine oxidase inhibitor gave contradictory results. However, because certain amino acids are known to possess an insulin-releasing effect, it is not improbable that such an effect of, e.g., L-5-HTP might mask the inhibitory action of the monoamine oxidase inhibitor. A recent report by Frohman (1971) showing that chronic treatment of rats with monoamine oxidase inhibitors resulted in decreased plasma insulin levels supports the above findings in mice (paper 11). Frohman (1971) also reported on an acute insulin release after administration of pargyline or mebanazine as previously reported after nialamide (Gagliardino *et al.* 1970 b). Furthermore, Frohman (1971) showed that the acute insulin release after pargyline was augmented by adrenaline. These monoamine oxidase inhibitors might possibly have slight α -blocking properties, which would explain their slight insulin releasing capacity, especially when combined with adrenaline (see paper 8).

In conclusion, the present studies (paper 11) have shown that, in the β -cell, the intracellular levels of monoamines such as 5-HT are able to modify insulin releasing processes *in vivo*. Injection of the amine precursor inhibited sulphonylurea-stimulated and L-IPNA-stimulated insulin release, which in the latter case could be totally suppressed by combination with a monoamine oxidase inhibitor. A monoamine oxidase inhibitor alone could partially inhibit insulin release by the above compounds. The inhibitory action of L-5-HTP was abolished by pretreatment with an inhibitor of aromatic amino acid decarboxylation. It is suggested that the amine itself is responsible for the effects obtained.

VI. ACID AMYLOGLUCOSIDASE AND INSULIN SECRETION

Amylolytic enzymes producing glucose through an exoamylolytic approach are known to be present in several species of fungi of the Aspergillus (Kerr, Cleveland and Katzbeck 1951, Pool and Underkofler 1953, Barker and Fleetwood 1957, Pazur and Ando 1959, amongst others) and Rhizopus groups (Tsujiisaka, Fukumoto and Yamamoto 1958, Phillips and Caldwell 1951, Pazur and Okada 1967, amongst others). Such enzymes have also been isolated from yeasts and bacteria (Hopkins and Kulka 1957, French and Knapp 1950, amongst others) and from plant sources (Takahashi and Shimomura 1968, Marshall and Taylor 1971, amongst others). The enzymes can be classified according to their preference for attacking either glycogen or maltose. Amyloglucosidases (preferentially attacking

glycogen or starch) act with inversion of the configuration of the reaction product, whereas α -glucosidases (preferentially attacking maltose) act with retention (cf. Reese, Maguire and Parrish 1968, paper 4). Furthermore, the amyloglucosidases are characterized by their ability to liberate glucose by the stepwise removal of glucose units from the non-reducing ends of glucose polymers (Pazur and Ando 1959). Mammalian tissues are also known to contain significant amounts of exoamylolytic hydrolases (cf. Smith, Taylor and Whelan 1968); these have been found in liver, muscle, kidney, spleen, and brain. The discovery of exoamylolytic activity in islet tissue (papers 5 and 13) raised the question of whether this activity was mainly to be referred to as an amyloglucosidase or as an α -glucosidase. The data of paper 5 showed that islet glycogen hydrolyzing activity and islet maltose hydrolyzing activity had different heat inactivation patterns, thus suggesting that both an amyloglucosidase and an α -glucosidase might be present. It was observed, however, that the exoamylolytic activity found in islet tissue preferentially attacked glycogen (unpublished) showing that the amyloglucosidase activity was predominant. Thus, the exoamylolytic activity recorded in islet tissue with the above-discussed method (paper 5) and the activity of the highly purified fungal enzyme (papers 4 and 12) can both be characterized as representing an acid amyloglucosidase.

The physiological significance of mammalian amyloglucosidases and α -glucosidases is largely unknown. The discovery by Hers (1963) that an acid α -1,4-glucosidase (and an acid amyloglucosidase ?) was not present in the liver, heart, and skeletal muscles of children with the fatal form of glycogenosis called Pompe's disease suggested that such enzymes might play a vital role in glycogen degrading processes. In spite of this discovery further investigations into the possible physiological significance of these acid hydrolases are largely lacking. As far as the author is aware, only reports by the Rosenfeld-group (cf. Rosenfeld, Popova and Orlova 1970) showing that exogenous adrenaline influenced exoamylolytic hydrolases in different tissues of the rat, and a recent report by Gennser, Lundquist and Nilsson (1971) who observed that the activity of acid amyloglucosidase in human foetal liver significantly increased during asphyxia, have dealt with the possible physiological implication of these enzymes.

In 1951, Toreson demonstrated that human and experimental diabetes mellitus was accompanied by glycogen infiltration in the pancreatic islets. Seventeen years later, Matschinsky and Ellerman (1968) showed that glycogen is a normal constituent of mammalian β -cells. They also reported that the glycogen phosphorylase activity in the islets of the insulin hypersecreting obese hyperglycaemic mouse (AO-mouse) was

very low. These findings and the hypothesis proposed by Samols, Marri and Marks (1966) that β -cell glycogenolysis may be the source of intracellular glucose or glucose metabolites acting as mediators for insulin release, suggested to me that the hydrolytic as opposed to the phosphorolytic breakdown of β -cell glycogen might be of physiological significance. This hypothesis is further strengthened by recent reports by Hellman, Idahl and Danielsson (1969) and Hellman and Idahl (1970) showing that insulin releasing agents, such as glucagon and the sulphonylurea compound glibenclamide, are capable of inducing β -cell glycogenolysis.

A. Effect of exogenous, purified fungal acid amyloglucosidase on insulin secretion

A preliminary attempt to evaluate the possible role of hydrolytic glycogenolysis in the β -cell for insulin secreting processes was based on the observation in man (Badhuin, Hers and Loeb 1964, Hug and Schubert 1967) that fungal amylases administered as crude extracts were able to penetrate into liver cells. Moreover, Cuthbertson, Fleming and Rice (1967) showed that a partially purified fungal acid amyloglucosidase injected into rats was able to lower liver glycogen stores. Thus the ability of an exogenously administered macromolecule, such as acid amyloglucosidase (molecular weight about 100,000), to penetrate into cells without appreciable loss of biological activity offered unique possibilities for studying its possible physiological significance. It was therefore considered worth while to administer a highly purified acid amyloglucosidase (papers 4 and 12) and to see whether it would penetrate also into the β -cell and consequently permit a preliminary answer to the question of whether hydrolytic breakdown of β -cell glycogen could be of physiological significance for insulin secreting processes. The data presented in paper 12 showed that administration of a highly purified acid amyloglucosidase to normal non-fasted mice induced a slight elevation of the plasma insulin levels, and a fall in blood glucose level, which invariably lasted for several hours. Moreover, alloxan diabetic animals in the β -cell regenerating phase, presumed to contain raised levels of β -cell glycogen as a consequence of their hyperglycaemic condition, were found to respond with markedly elevated plasma insulin levels and decreased blood glucose levels upon enzyme administration. These findings, together with the observation (paper 14) that islet amyloglucosidase activity in normal mice increased by about 30 % 3 h after enzyme administration, suggested that the enzyme indeed penetrated into the β -cells with preserved biological activity. The possibility that the rise

in amyloglucosidase activity seen in the β -cell might be due not to the presence of the enzyme within the cells, but rather to the presence of the enzyme in residual blood, tissue fluid, or on the β -cell surface, was also considered. However, this is unlikely, because the amyloglucosidase activity in serum had returned to control values at least 2 h before islet isolation (paper 12). Furthermore, an effect on the cell membrane would not be expected to occur 3 h after enzyme injection, because the half-life of the enzyme in blood is only about 18 min (paper 12) and the elevation of plasma insulin levels was not recorded until 2.5 - 3 h after enzyme administration. If the administered enzyme was not located within the β -cell lysosomes, then the "free" activity of the osmotically protected islet homogenate would be considerably augmented, which was not the case (unpublished). The preserved activity of the enzyme after penetration into the β -cell and its possible implication in insulin releasing processes was further supported by the observation (paper 14) that pretreatment with acid amyloglucosidase 3 h before the experiment markedly augmented glibenclamide-induced insulin release.

The half-life of the intravenously injected enzyme in blood (18-19 min) was unaltered whether the enzyme was injected into normal mice or into alloxan diabetic animals that had been diabetic for 5 months (paper 12). Moreover, the liver glycogen depleting effect of the acid amyloglucosidase in alloxan diabetic mice that had been diabetic for about 2 months was as pronounced as in normal mice. These observations showed that the ability of the enzyme to penetrate through the vascular walls and through the liver cell membrane was the same in controls and in animals that had been severely diabetic for a considerable time.

B. Endogenous activity of acid amyloglucosidase in pancreatic β -cells

To further elucidate the possible role of acid amyloglucosidase in insulin secreting processes, an investigation was undertaken to determine acid amyloglucosidase activity in isolated pancreatic islets of the mouse (paper 13). It was observed that amyloglucosidase activity in collagenase-isolated pancreatic islets of the normal NMRI-mouse displayed a pH-optimum around pH 5.0 - 5.5. The enzyme could thus be characterized as an acid amyloglucosidase. The activity of acid amyloglucosidase at pH 5.0 in islet tissue was twice that of liver and 10 times that of skeletal muscle. The finding that the acid amyloglucosidase activity in islet tissue from alloxan diabetic mice was markedly reduced, to about 10 % of normal level, suggests that the activity is mainly localized in the β -cell. The hypothesis that the hydrolytic breakdown of β -cell glycogen plays a role for insulin releasing processes was strengthened by the observation (paper 13)

that the acid amyloglucosidase activity in islets of the insulin hyper-secreting obese mouse (AO-mouse) was more than twice that of its lean litter mates or the normal NMRI-mouse.

C. Interaction of monoamines with acid amyloglucosidase

It has been known for several years from *in vitro* experiments that certain glycoside hydrolases are inhibited by Tris (Larner and Gillespie 1956, Halvorsen and Ellias 1958, Dahlqvist 1961, Jørgensen 1963). There is some dispute whether the inhibitory effect of Tris is a function of its amino-group or its hydroxyl groups (cf. Kelemen and Whelan 1965, Dahlqvist 1961). With regard to the purified fungal acid amyloglucosidase from Aspergillus niger, it was thought probable that the inhibition by Tris at neutral pH is due to the amino-groups, because both Tris and other amines, such as cetylpyridinium chloride and protamine hydrochloride, were found to be potent inhibitors at pH 7.0 (paper 4). As glibenclamide-induced insulin release was considerably enhanced by the previous treatment with purified acid amyloglucosidase (paper 14), whereas pretreatment with an amine precursor (L-5-HTP) or a monoamine oxidase inhibitor (paper 11) significantly inhibited glibenclamide-stimulated insulin release, the question arose whether an interaction between monoamines and acid amyloglucosidase in the β -cell could possibly contribute to an explanation of the latter observation. It was observed (paper 14) that the marked enhancement of glibenclamide-induced insulin release after enzyme pretreatment was impaired after treatment with the monoamine precursors L-5-HTP and L-DOPA and also after treatment with a monoamine oxidase inhibitor. The combined action of L-DOPA and a monoamine oxidase inhibitor caused the most marked inhibition, although it did not totally suppress the glibenclamide-induced insulin release. The failure of the latter treatment to abolish glibenclamide-induced insulin release is in accordance with the observation that part of the insulin pool which can be released by the sulphonylurea compound is not affected by the amine (paper 11). As the amine is probably associated with the specific secretory granules (paper 10), it is tempting to speculate that the "amine-independent" insulin release is achieved through the alternative insulin secretion route which is presumed to by-pass the secretory granules (Like and Miki 1967, Renold 1970). Model experiments in the test tube (paper 14) showed that dopamine and 5-HT are capable of significantly inhibiting the action of the purified acid amyloglucosidase at neutral pH (pH 7.2), whereas no inhibition was recorded at pH 5.0, which suggests that the actual intracellular pH is of decisive importance for the inhibitory action of the amines. Thus, shifts in intracellular pH (alteration of lysosomal integrity?) might

regulate the inhibitory effect elicited by the intracellular amines through their action on acid amyloglucosidase.

D. The mechanism of action of sulphonylurea -induced insulin secretion.
Lysosomes and insulin secretion

The amyloglucosidase activity in isolated pancreatic islets was found to display sedimentability ($19.500 \times g$ for 10 min), structure-linked latency, and an acid pH-optimum (paper 13) (for definitions, cf. Vaes 1966). These properties are inherent in the concept of lysosomes and suggest a possible lysosomal localization of islet acid amyloglucosidase, which points to an implication of lysosomes in insulin secreting processes. Being a macromolecule, the exogenous acid amyloglucosidase presumably enters the β -cell through endocytosis. The endocytic vesicle would be incorporated into a lysosome and the enzyme activity retained. The action of the endogenous enzyme or the exogenous enzyme, which is taken up and stored by the cell, could thus be expected to involve the lysosomes. Pretreatment with the purified acid amyloglucosidase was found to markedly enhance sulphonylurea-induced insulin release. Moreover, it was observed that there was a dose dependent increase in "free" activity of the enzyme 2 min after glibenclamide injection (paper 14). The "free" activity of islet acid amyloglucosidase in control animals was found to be remarkably constant from experiment to experiment (papers 13 and 14); this increase was therefore interpreted as reflecting an enhancement of lysosomal permeability after sulphonylurea treatment. The role of lysosomes in sulphonylurea-induced insulin release was further studied with the use of different steroids as lysosomal stabilizers and labilizers (cf. Weissmann 1969). It was observed (paper 14) that glibenclamide-induced insulin release was enhanced after pretreatment with progesterone, a lysosomal labilizer, but diminished after pretreatment with dexamethasone, a lysosomal stabilizer.

Gylfe (1971) showed that glibenclamide added in vitro increases the release of particle-bound islet β -glucuronidase and acid phosphatase in crude lysosomal fractions from pancreatic islets of obese hyperglycaemic mice. Hellman, Idahl and Danielsson (1969) reported that the compound induced β -cell glycogenolysis; Howell and Lacy (1969) showed that labelled glibenclamide penetrated into intact islet cells. All this evidence suggests that sulphonylurea-stimulated insulin release is characterized by the following events in the β -cell: Lysosomal activation - Increase of the "free" activity of the lysosomal acid amyloglucosidase - Hydrolytic glycogen breakdown through acid amyloglucosidase yielding glucose - Insulin release.

The possibility that the secretory granule itself is provided with intragranular lysosomal structures or lysosomal enzymes is unlikely, because isolated β -granules are stable in the presence of sulphonylureas (Coore et al. 1969, Howell, Young and Lacy 1969) which labilize islet lysosomes both in vitro (Gylfe 1971) and in vivo (paper 14). Moreover, several physicochemical properties of insulin secretory granules clearly differ from those generally ascribed to lysosomes (Coore et al. 1969, Howell, Young and Lacy 1969, Lambert et al. 1970, Beaufay 1969, Lucy 1969). An electron-microscope study failed to demonstrate acid phosphatase, an enzyme predominantly present in lysosomes, in the secretory granules of rabbit β -cells (Lazarus, Volk and Barden 1966). A similar study of rat β -cells recently published (Orci et al. 1971), however, showed this enzyme to be present in a number of secretory granules. This finding raises the question of whether a certain lysosome population may be closely associated with the secretory granules. There are several morphological observations connecting the secretory granules of polypeptide producing endocrine cells with lysosomal "autophagy" showing lysosomal granulolysis in the degradation of undischarged secretory products (Smith and Farquhar 1966, Orci et al. 1968, Creutzfeldt et al. 1969, Boquist 1970). De Duve (1969) proposed that such processes should be designated crinophagy. From the above-discussed data; there seems to be good evidence that β -cell lysosomes are indeed implicated in insulin releasing processes and not only involved in the disposal of excess secretory products.

VII. SUMMARY

The influence of extracellular monoamines, β -cell monoamines, and acid amyloglucosidase on insulin secretion was investigated in mice. In addition, the insulin secretory capacity and the carbohydrate balance during the developmental and manifest stage of alloxan diabetes was studied in order to obtain models resembling various diabetic conditions. Methods for glycogen determination, purification of fungal acid amyloglucosidase, and determination of acid amyloglucosidase in isolated pancreatic islets were developed. Serial blood sampling in unanaesthetized mice was applied for the purpose of glucose determination.

The results obtained were interpreted as follows:

Acute diabetogenesis after alloxan is characterized by an initial hyperglycaemic phase due to marked liver glycogenolysis and cessation of insulin secretion. The ensuing hypoglycaemic phase is due to uncontrolled insulin leakage by the damaged β -cells. Manifest alloxan diabetes is characterized by absence of or severely impaired insulin secretion, decreased levels of liver and muscle glycogen, and high concentrations of blood glucose. Diabetic animals in the β -cell regenerating phase are characterized by relatively high blood glucose levels and a significant number of functioning β -cells.

Adrenaline has a dual action on insulin secretion analogous to its effect on the vascular system. During basal conditions, adrenaline will stimulate insulin secretion through its β -adrenergic properties, whereas elevated levels of the amine have an inhibitory action through its α -adrenergic properties. Intracellular monoamines in the β -cell are associated mainly with the insulin secretory granules. They are able to inhibit insulin secreting processes, possibly through the interaction of the monoamine with an enzyme, acid amyloglucosidase, shown in the present study to be present in the β -cell. This hydrolytic enzyme displays sedimentability, structure-linked latency, and an acid pH-optimum. These properties are interpreted as indicating a lysosomal localization of the enzyme. Acid amyloglucosidase is implicated in insulin secreting processes through its ability to liberate glucose from β -cell glycogen. Its activity in isolated pancreatic islets from alloxan diabetic mice is about 10 % of that from normal mice. On the other hand, in insulin hypersecreting obese mice, the activity of the enzyme is more than twice that of normal mice.

Highly purified fungal acid amyloglucosidase given to mice is taken up by the β -cell and stored in the lysosomes. Administration of the enzyme to normal mice or alloxan diabetic mice in the β -cell regenerating phase results in an elevation of basal plasma insulin levels. Moreover, pretreatment of normal mice with the purified enzyme results in a marked enhancement of sulphonylurea-induced insulin release.

A sulphonylurea compound augments the proportion of "free" activity of islet acid amyloglucosidase after administration in vivo. Stimulation of insulin release by a sulphonylurea compound is decreased by pretreatment with a lysosomal stabilizer, but increased by a lysosomal labilizer.

It is suggested that β -cell lysosomes are implicated in certain insulin releasing processes evoked through the following events in the β -cell:
Lysosomal activation - Increase of the "free" activity of acid amyloglucosidase - Hydrolytic glycogen breakdown through acid amyloglucosidase yielding glucose - Insulin release.

VIII. ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Professor Stephen Thesleff, head of this department, who kindly placed laboratory facilities to my disposal, and who has read and criticized the manuscript with great care, to Dr. Claus Rerup, who has been my teacher and under whose guidance it has been my privilege to work, to Professor Ragnar Ekholm and Drs. Rolf Håkanson, Lars Ericson, Kai Lindstrand, Jan Börjeson, Jan-Owe Josefsson and Christer Owman for fruitful co-operation and stimulating discussions, to Other colleagues in the Department of Pharmacology for stimulating discussions and encouraging interest, to Dr. John Harris, Department of Neurology, University of Newcastle, School of Medicine, for careful reading and correction of my papers, to Mrs. Lena Kvist, Miss Gertie Malmstein, Miss Siw Erman, Miss Monica Johansson, Miss Anita Åkesson, Miss Ann-Christin Helander, Mrs. Karin Irenesson, Miss Christina Lilja and Miss Margareta Lönnerberg for their skilful technical assistance, and to The Medical Faculty, University of Lund, the Swedish Diabetes Association, the Swedish Society for Medical Research, "Vera och Carl J. Michaelsens donationsfond", and "Dr. Martha P:son Hennings donationsfond" for financial support of the investigations.

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